J. Physiol. (1977), **273**, *pp.* 443–457
With 3 plates
Printed in Great Britain

ACTION OF BROWN WIDOW SPIDER VENOM AND BOTULINUM TOXIN ON THE FROG NEUROMUSCULAR JUNCTION EXAMINED WITH THE FREEZE-FRACTURE TECHNIQUE

BY D. W. PUMPLIN AND T. S. REESE

From The Laboratory of Neurobiology, University of Puerto Rico, Medical Science Campus, San Juan, Puerto Rico and Laboratory of Neuropathology and Neuroanatomical Sciences, NINCDS, National Institutes of Health, Bethesda, Maryland, U.S.A.

(*Received 21 February 1977*)

SUMMARY

1. Structural changes which normally accompany transmitter release at frog neuromuscular junctions are visualized with the freeze-fracture technique. The effects of brown widow spider venom and botulinum toxin were evaluated in terms of their ability to block or produce these structural changes. Changes produced by these neuropoisons were correlated with their known effects on neurotransmitter release.

2. Fusion of synaptic vesicles with the presynaptic plasmalemma, normally evoked by electrical stimulation, was abolished at neuromuscular junctions from frogs treated with botulinum toxin.

3. The concentration of large intramembranous particles in the presynaptic plasmalemma, an indication of the excess of synaptic vesicle fusion over recovery of synaptic vesicle membrane, was increased by treatment with brown widow spider venom, even in the presence of botulinum toxin.

4. When external calcium was present, sites of vesicle fusion induced by brown widow spider venom, as well as by electrical stimulation, were located mainly in the active zone. In the absence of external calcium, many plasmalemmal deformations, also thought to be sites of vesicle fusion, were more evenly dispersed over the presynaptic surface of nerve terminals.

5. Botulinum toxin decreased the number of vesicle fusion sites in the active zone induced by spider venom in the presence of external calcium but had little effect on the number of fusion sites induced by spider venom in the absence of external calcium.

6. Nerve terminals soaked in a sodium-free Ringer solution were

partially depleted of vesicles. Addition of spider venom to this Ringer did not cause additional depletion of vesicles.

7. Formation of cation-permeable channels in the presynaptic membrane could account for these effects of spider venom on the frog neuromuscular junction. Botulinum toxin blocks vesicle fusion by some means which is not yet understood.

INTRODUCTION

Use of the freeze-fracture technique has led to new insights into processes occurring at synapses. The method exposes more extensive views of plasma membranes than are readily obtainable with conventional thin-sectioning techniques and also exposes internal surfaces of membranes which are inaccessible with other techniques. Ultrastructural changes revealed by the freeze-fracture technique which occur concomitant with the evoked release of neurotransmitter have been described at synapses (Akert, Pfenninger, Sandri & Moor, 1972; Pfenninger, Akert, Moor & Sandri, 1972; Pfenninger & Rovainen, 1974) and neuromuscular junctions (Dreyer, Peper, Alkert, Sandri & Moor 1973; Heuser, Reese & Landis, 1974). In brief, these consist of the appearance of deformations in the presynaptic plasmalemma, considered to represent synaptic vesicles fusing with this membrane during the period of fixation, and in a rise in the concentration of large particles (10–12 nm diameter) in the cytoplasmic face of the plasmalemma, thought to be particles contained in the membranes of synaptic vesicles that are transferred to the presynaptic plasmalemma during vesicle fusion (Heuser & Reese, 1975). We have used the freeze-fracture method to investigate further the effects on neurotransmitter release of two neuropoisons, brown widow spider venom and botulinum toxin, hoping to gain more knowledge of the mechanism of action of these poisons.

Extracts of the venom glands of brown widow spiders (*Latrodectus geometricus*) are immunologically indistinguishable from extracts of venom glands of black widow spiders (*L. mactans*) (McCrone & Netzloff, 1965), and have similar physiological effects. Both cause a massive, but limited, release of neurotransmitters and a concomitant depletion of synaptic vesicles from nerve terminals (Clark, Mauro, Longenecker & Hurlbut, 1970; Longenecker, Hurlbut, Mauro & Clark, 1970; Pumplin & del Castillo, 1975). These toxic effects of black widow venom are associated with a protein component of molecular weight 125,000 which has been partially purified by two different methods (Frontali, Ceccarelli, Gorio, Mauro, Siekevitz, Tzeng & Hurlbut, 1976 and Grasso, 1976; Ornberg, Smyth & Benton, 1976). Recently a purified fraction has been shown to enter artificial bilayer membranes and form long-lived cation-conductive channels (Finkelstein, Rubin & Tzeng, 1976).

Botulinum toxin, a protein of molecular weight 150,000, blocks neurotransmission at cholinergic synapses (for review see Simpson, 1971). Conduction of the action potential into the nerve terminals is not affected (Harris & Miledi, 1971); muscles remain sensitive to applied ACh and toxin-treated terminals contain approximately normal concentrations of ACh (Thesleff, 1960). Miniature end-plate potentials persist after treatment with botulinum toxin, but both their frequency and amplitude are much reduced (Harris & Miledi, 1971; Spitzer, 1972). This finding led to the speculation that the toxin inhibited the filling of synaptic vesicles with ACh (Boroff, del Castillo, Evoy & Steinhardt, 1974), which would also explain its specificity for cholinergic synapses. However, widow spider venoms induce release of normal-sized quanta of transmitter from botulinum-blocked terminals in which electrical depolarization elicits no release (del Castillo & Pumplin, 1975; Cull-Candy, Lundh & Thesleff, 1976).

METHODS

Cutaneous pectoris muscles with short lengths of nerve attached were removed from small frogs (*Rana pipiens*), and pinned to a layer of Sylgard in a Petri dish. Muscles were bathed in the appropriate Ringer solution 30–60 min before the application of electrical stimulation or venom. Glass micropipettes filled with 3 M potassium chloride were used for recording end-plate potentials.

Brown widow spider venom was prepared by homogenizing the venom glands of five spiders in 1 ml. Ringer solution. The homogenate was centrifuged for 30 sec in a Beckman Microfuge and the supernatant solution applied to the muscle. After 5–10 min, a large volume of fixative (3% formaldehyde in the appropriate Ringer) was added. After 5 additional minutes, the formaldehyde solution was replaced by 1% glutaraldehyde in the appropriate Ringer. Sections of the fixed muscle containing neuromuscular junctions were cut out, treated with 25% glycerol, frozen in liquid Freon-22, and subsequently fractured without etching in a Balzers 360 M freeze-fracture apparatus. Fractured surfaces were shadowed with platinum from an electron beam gun and coated with carbon. Replicas of fractured surfaces were cleaned in sodium hypochlorite (Purex) and examined in an AEI-802 electron microscope operated at 80 kV.

For microscopy of thin sections, muscles were either fixed in 1% glutaraldehyde in 0·05 M-Tris buffer, pH 7·2, or in the same fixatives used to prepare them for freeze-fracturing. They were postfixed in 1% OsO_4, block stained in 1% uranyl acetate, dehydrated, and embedded in Araldite (Ciba).

For electrical stimulation, muscles were curarized (curare 10^{-5} g/l. in Ringer) to avoid contraction. They were stimulated at 20 Hz with supramaximal pulses for 5 min and simultaneously fixed in 3% formaldehyde in Ringer. Further preparation for freeze-fracture was as described above.

For studies on botulinum toxin, frogs were injected in the dorsal lymph sac with 0·1 ml. (nominally $3·5 \times 10^6$ mouse LD_{50}) of a solution of crystalline Type A toxin in 0·05 M-sodium acetate buffer, pH 4·5. After 18–24 h, the frogs were paralysed. Cutaneous pectoris muscles were removed and tested as described above. Nerve action potentials were readily recorded, but evoked endplate potentials were never found, although the resting potentials of the muscle fibres were within the normal range. Electrical stimulation and treatment with venom were performed as described above.

The normal Ringer solution (Ca-Ringer) contained (mM) Na^+, 111; K^+, 2; Ca^{2+}, 1·8; HEPES, 5; dextrose, 3. The calcium-free Ringer solution contained (mM) Na^+, 109; K^+, 2·5; Ca^{2+}, 0; Mg^{2+}, 4; EGTA, 1; HEPES, 5. The sodium-free, calcium-free Ringer contained (mM) glucosamime-HCl, 72; Tris-OH, 23·5; Mg^{2+}, 4, EGTA, 1. Solutions were adjusted to pH 7·2 and 220 m-osmole measured by freezing-point depression. All experiments were performed at 20–22 °C.

Measurements

Micrographs were taken at 27,400 × and printed at a nominal over-all magnification of 76,700. Active zones and plasmalemmal deformations were clearly defined in most micrographs. The distance between the centre of each deformation and the centre of the nearest ridge was measured along a line normal to the ridge. On the basis of histograms of the distribution of such distances (Heuser *et al.* 1974), deformations were considered to occur *at* the active zone if this distance was less than 110 nm (approximately two synaptic vesicle diameters). Means and variances of these differences were determined for each terminal (2–30 active zones).

Concentrations of large particles were measured in micrographs in which the active zones were well defined, the membrane surface relatively flat, and the shadowing of particles even throughout. Measurements were limited to portions of the membrane surface lying between two adjacent active zones and excluded plasmalemmal deformations. Measurements were made by a person who did not know the treatment protocol. Only particles as large as, or larger than, those in the inner row at the ridge were included.

Statistics

The variances of the measurements of large particle concentrations, distribution of plasmalemmal deformations, and nunbers of synaptic vesicles per terminal, were not uniform among the various treatments, which made normal analysis of variance invalid. Therefore, we used the non-parametric Kruskal-Wallis test, followed by multiple comparisons between pairs of treatments using a large sample approximation (Hollander & Wolfe, 1973).

RESULTS

Botulinum toxin

When control terminals were stimulated electrically during fixation, numerous deformations appeared in the plasmalemma (Pl. 1, fig. 1), confirming previous results (Heuser *et al.* 1974). These deformations are considered to be sites of endo- and exocytosis. In our experiments, a relatively slow fixative, 3% formaldehyde, was used to increase the time of fixation and thereby increase the number of plasmalemmal deformations caught by the fixation (Heuser *et al.* 1974). In muscles obtained from frogs paralysed by prior injection of botulinum toxin, electrical stimulation to the afferent nerve did not give rise to endplate potentials. When such muscles were stimulated in this way during formaldehyde fixation, very few plasmalemmal deformations were found (Pl. 1, fig. 2; Table 1*a*).

Electrical stimulation of control terminals produced an increase in large particles in the presynaptic plasmalemma (Table 2) confirming

previous results (Heuser & Reese, 1975). These large particles are thought to be contributed by synaptic vesicles which have fused with the plasmalemma. The concentration of large particles in terminals poisoned by botulinum toxin and stimulated electrically was not increased over that found in unstimulated terminals (Table 1*b*). These two results show directly that little or no exocytosis occurs in response to electrical stimulation of botulinum toxin treated terminals and indicate that the toxin affects transmitter release itself, in addition to possible effects on the filling of synaptic vesicles with acetylcholine (Boroff *et al.* 1974).

TABLE 1. Effects of botulinum toxin on electrically stimulated neuromuscular junctions

a Plasmalemmal deformations

Treatment	Deformations/μm ridge ± S.E. of mean (no. of terminals)
ES-Ca[1]	7·12 ± 0·90 (19)
ES-Ca-BotTX[2]	0·08 ± 0·03 (8)

b Concentration of large particles

Treatment	Particles/μm^2 ± S.E. of mean (no. of micrographs[5])	Average rank	*P* of significance of difference from[6]: (ES-Ca-BotTX)	(ES-mg)	Rest
ES-Ca[1]	139·4 ± 6·2 (9)	34·8	< 0·01	< 0·001	< 0·001
ES-Ca-BotTX[2]	43·7 ± 4·2 (11)	15·5	—	n.s.	n.s.
ES-Mg[3]	48·8 ± 5·9 (10)	17·8	—	—	n.s.
Rest[4]	43·3 ± 10·9 (9)	13·1	—	—	—

1. Motor nerve stimulated supramaximally at 20 Hz for 5 min while muscle was immersed in 3% formaldehyde fixative in calcium-containing Ringer solution. See Methods section for further details.

2. Stimulation in fixative as in 1. Muscle obtained from frog injected 18–20 hr earlier with $3{\cdot}5 \times 10^6$ mouse LD_{50} Type A botulinum toxin.

3. Stimulation in fixative as in 1 performed in calcium-free Ringer solution.

4. Muscle fixed in the absence of electrical stimulation.

5. Number of micrographs from which particle density was measured in two or three areas of plasmalemma (2–3 μm^2 total area/print). Measurements were made on several different terminals for each treatment.

6. Calculated from non-parametric test employing ranked data. See Statistics section.

Brown widow spider venom – large particles

When frog muscles were soaked in brown widow spider venom in normal Ringer at 1–2 °C, which does not lead to an increase in the m.e.p.p. frequency (D. W. Pumplin, unpublished), nerve terminals were not depleted of

vesicles and the density of large particles remained low (Pl. 2, fig. 3). However, when venom was applied at room temperature, both vesicle depletion and increases in the density of large particles occurred (Table 2). Increases in the concentrations of large particles indicate that the venom caused a net excess in the rate of exocytosis over that of endocytosis, thereby increasing the net incorporation of synaptic vesicle membrane, bearing the large particles, into the presynaptic plasmalemma (Heuser & Reese, 1975). The

TABLE 2. Relative concentrations of large particles in the presynaptic plasmalemma of nerve terminals treated with BrWSV

Treatment	Particles/μm^2 ± S.E. of mean (no. of micrographs[4])	Average rank	P of significance of difference from[5]: V-Mg	V-Mg-BotTX	V-Ca-BotTX	Rest
V-Ca[1]	273·3 ± 18·3 (9)	84.2	n.s.	< 0·01	< 0·05	< 0·001
V-Mg[2]	177·7 ± 13·8 (20)	64·6	—	n.s.	n.s.	< 0·001
V-Mg-BotTX[3]	120·8 ± 13·8 (24)	48·2	—	—	n.s.	< 0·01
V-Ca-BotTX[3]	90·4 ± 8·5 (9)	43·4	—	—	—	n.s.
Rest	45·3 ± 4·0 (30)	21·8	—	—	—	—

1. BrWSV homogenate of venom glands of five spiders applied in calcium-Ringer solution 5–10 min before formaldehyde fixation. See text for details.

2. BrWSV applied in calcium-free Ringer solution.

3. BrWSV applied in appropriate Ringer solution to muscles of frogs treated 18–20 hr earlier with $3·5 \times 10^6$ mouse LD_{50} of Type A botulinum toxin.

4. Particles were counted in two to three areas of plasmalemma (approximately 1 μm^2 each) from each micrograph. Portions of at least three different terminals were counted in each experiment.

5. Calculated from non-parametric test employing ranked data. See Statistics section.

increased concentration of large particles induced by BrWSV occurred in the presence or absence of external calcium (Table 2), in agreement with the electrophysiological effects of the venom (Longenecker, Hurlbut, Mauro & Clark, 1970). However, the increased concentration obtained with comparable amounts of venom was depressed in the absence of external calcium or after botulinum toxin treatment, in accord with previous observations that the rate of release of ACh from rat sympathetic ganglion induced by a given amount of black widow spider venom was depressed by these treatments (Pumplin & McClure, 1977).

Brown widow spider venom – plasmalemmal deformations

When muscles were treated with brown widow spider venom and fixed during the resultant period of rapid release of neurotransmitter, manifested by a high frequency of miniature endplate potentials, many

plasmalemmal deformations were seen in freeze-fractured nerve terminals. The deformations themselves were indistinguishable from those obtained by fixation during electrical stimulation. Increased numbers of plasmalemmal deformations were found both in both normal and calcium-free Ringer solution, in agreement with previous findings that the venom increased the frequency of m.e.p.p.s. in the absence of external calcium (Longenecker *et al.* 1970).

TABLE 3. Relative frequencies of plasmalemmal deformations in the presynaptic plasmalemma of nerve terminals treated with BrWSV. See Table 2 for explanation of treatments

a Deformations < 110 nm from the centre of the ridge (in active zone)

Treatment	Deformations/ μm of ridge ± S.E. of mean (no. of terminals)	Average rank	*P* of significance of difference from: V-Mg	V-Mg-BotTX	V-Ca-BotTX
V-Ca	10·16 ± 1·50 (12)	55·6	< 0·001	n.s.	< 0·001
V-Mg	2·54 ± 0·60 (21)	26·2	—	n.s.	n.s.
V-Mg-BotTX	4·10 ± 0·42 (21)	40·0	—	—	< 0·05
V-Ca-BotTX	1·82 ± 0·64 (14)	20·8	—	—	—

b Deformations > 110 nm from the centre of the ridge (outside active zone)

Treatment	Deformations/ μm of ridge ± S.E. of mean (no. of terminals)	Average rank	*P* of significance of difference from: V-Mg	V-Mg-BotTX	V-Ca-BotTX
V-Ca	0·61 ± 0·22 (12)	22·8	< 0·05	n.s.	n.s.
V-Mg	2·20 ± 0·36 (21)	46·7	—	n.s.	< 0·05
V-Mg-BotTX	1·42 ± 0·26 (21)	38·4	—	—	n.s.
V-Ca-BotTX	0·81 ± 0·47 (14)	20·5	—	—	—

c Total deformations

Treatment	Deformations/ μm of ridge ± S.E. of mean (no. of terminals)	Average rank	*P* of significance of difference from: V-Mg	V-Mg-BotTX	V-Ca-BotTX
V-Ca	10·76 ± 1·51 (12)	52·7	< 0·05	n.s.	< 0·001
V-Mg	4·73 ± 0·78 (21)	31·2	—	n.s	n.s.
V-Mg-BotTX	5·52 ± 0·51 (21)	37·7	—	—	< 0·05
V-Ca-BotTX	2·63 ± 0·82 (14)	19·0	—	—	—

However, the locations of venom-induced plasmalemmal deformations relative to the active zone were strikingly dependent on the presence or absence of external calcium (Table 3). In calcium-containing Ringer solution, venom-induced deformations were located almost entirely at the active zone (Pl. 2 fig. 4), while in calcium-free Ringer solution, deformations

occurred both at and outside the active zone with approximately equal frequency (Pl. 3, fig. 5). Prior treatment with botulinum toxin depressed the number of deformations at the active zone induced by BrWSV in normal Ringer solution but had little effect on venom-induced deformations in calcium-free Ringer solution (Table 3).

Plasmalemmal deformations occurring in the active zone during transmitter release are thought to correspond to openings of synaptic vesicles. However, coated invaginations of the plasmalemma, which are the first step in endocytosis, can also produce plasmalemmal deformations outside the active zone. It has not been consistently possible to distinguish synaptic vesicle openings from coated invaginations on the basis of their size or particle distribution (Heuser *et al.* 1974). Therefore, we attempted to determine the nature of the plasmalemmal deformations outside the active zone by examining thin sections of nerve terminals. In a preliminary study, we examined 81 cross-sections of terminals treated with BrWSV in calcium-free Ringer solution. The average section thickness was 0·07 μm and can be assumed to be perpendicular to the long axis of the nerve terminal. Since there are approximately 1·4 μm of ridge per μm of nerve terminal (J. E. Heuser & T. S. Reese, unpublished), we have explored an area of plasmalemma equivalent to that adjacent to approximately 8 μm of ridge and found only two coated invaginations. However, if the plasmalemmal deformations which occurred outside the active zone in nerve terminals treated with venom in calcium-free Ringer represented endocytosis, we should have found more than eighteen coated invaginations. Thus, there may be a large discrepancy between the numbers of coated invaginations and the numbers of plasmalemmal deformations outside the active zone. We did not attempt a similar correlation for synaptic vesicle openings because, lacking a coat, they are difficult to identify with certainty in thin sections.

The rate of transmitter release, as represented by the total number of plasmalemmal deformations per length of ridge, was depressed in calcium-free Ringer or after treatment with botulinum toxin (Table 3*c*), in agreement with the absence of the usual increase in the concentration of particles and the depression in the rate of acetyl-choline output (Pumplin & McClure, 1977).

Venom activity in Na-free, Ca-free Ringer

Finkelstein *et al.* (1976) proposed that exposure of frog muscle to venom of the Italian black widow spider (*L. mactans tredecimguttatus*) in sodium-free, calcium-free Ringer would test whether the venom acted by affecting the permeability of the endplate plasmalemma to either of these ions. Their preliminary finding was that this treatment reduced the release

of acetylcholine when preparations were subsequently placed in additional venom in normal Ringer.

In our experiments, muscles were soaked for 1 hr in sodium-free, calcium-free Ringer, then soaked either in brown widow spider venom in this Ringer, or in the Ringer alone, for an additional hour, before preparation for electron microscopy. Random cross-sections of nerve terminals were photographed so that vesicles in the cross-sections could be counted. The results (Table 4) indicate that sodium-free Ringer itself causes a depletion of vesicles from nerve terminals, probably due to an increased inward flux of calcium as a result of decreasing the external sodium (Baker, Blaustein, Hodgkin & Steinhardt, 1967). Brown widow spider venom in sodium-free Ringer did not further deplete the terminals of vesicles.

TABLE 4. Vesicle content of nerve terminals treated with BrWSV in sodium-free, calcium-free Ringer solution

Treatment	No. of synaptic vesicles/ cross-section ± S.E. of mean (no. of cross-sections)	Average rank
Control Ringer[1]	$94{\cdot}1 \pm 9{\cdot}7$ (42) ***	97·8
0-Na^+ Ringer[2]	$28{\cdot}5 \pm 4{\cdot}0$ (32) n.s.	62·2
BrWSV; 0-Na^+ Ringer	$21{\cdot}3 \pm 5{\cdot}4$ (18) ***	50·8
BrWSV; control Ringer	$4{\cdot}0 \pm 1{\cdot}1$ (32)	23·0

1. NaCl, 123 mM; KCl, 2 mM; $MgCl_2$, 4 mM; EGTA, 1 mM; HEPES, 5 mM; pH 7·2, 220 m-osmole.

2. NaCl replaced by glucosamine-HCl, 72 mM; Tris-OH, 23·5 mM; pH 7·2, 220 m-osmole. Muscles were treated 2 hr in Ringer alone, or 1 hr in Ringer followed by 1 hr in venom in same Ringer.

3. ***, $P < 0{\cdot}001$; n.s., not significant. Calculated from non-parametric test employing ranked data. See Statistics section.

DISCUSSION

A purified component of black widow spider venom has been shown to enter lipid bilayers and form long lived channels which are highly permeable to cations (Finkelstein *et al.* 1976). We believe that formation of such channels in nerve terminal plasma membranes can account for the morphological effects we have observed. In sodium-containing Ringer, the increased permeability to sodium would depolarize the terminal. Such a depolarization is known to increase the frequency of m.e.p.p.s. if calcium is present externally (del Castillo & Katz, 1954). The transmitter release which follows depolarization produced by electrical nerve stimulation is manifested in the plasmalemma of the nerve terminal by rows of plasmalemmal deformations in active zones; each plasmalemmal deformation is the opening of a synaptic vesicle (Dreyer *et al.* 1973; Heuser *et al.* 1974).

We take the presence of a similar distribution of plasmalemmal deformations in the active zones of nerve terminals treated with spider venom (Pumplin & Reese, 1976*a*, *b*; Ceccarelli, Peluchetti, Grohovaz & Iezzi, 1976) to indicate that spider venom-induced transmitter discharge depends on depolarization-induced opening of synaptic vesicles. The parallel increases in the concentration of large plasmalemmal particles in nerve terminals treated with spider venom or electrically stimulated via their nerves (Heuser & Reese, 1975) also indicates a common mode of vesicle discharge.

In calcium-free Ringer, depolarization does not lead to transmitter release (del Castillo & Engbaek, 1954) nor are plasmalemmal deformations produced by electrical stimulation (Heuser *et al.* 1974). Nevertheless, channels made by the venom would allow sodium to move down its concentration gradient to enter the nerve terminal. The increased internal sodium concentration might then displace bound calcium from mitochondria (Lowe, Richardson, Taylor & Donatsch, 1976) or other internal stores (Kendrick, Blaustein, Fried & Ratzlaff, 1977). The resultant rise in free calcium concentration would increase the rate of transmitter release. Displacement of bound calcium by sodium is in accord with our observation that application of spider venom in calcium-free Ringer results in exocytosis, as manifested by numerous plasmalemmal particles. A displacement mechanism would also account for our failure to observe venom-induced vesicle depletion in sodium-free medium. Calcium chelators which may enter cells inhibit the action of spider venom, even when it is applied in calcium-free media (Ornberg & Smyth, 1976). This observation further supports the idea that a rise in the concentration of free calcium within the nerve terminal mediates venom-induced transmitter release even though calcium is absent externally.

The general rise in free calcium postulated above might also account for our finding that numerous plasmalemmal deformations were found outside the active zone in terminals treated with venom in the absence of external calcium. However, the nature of these plasmalemmal deformations is not yet clear because there are no consistent morphological criteria to distinguish exo- from endocytosis (Heuser *et al.* 1974). We suspect that many of these deformations indeed reflect exocytosis, for three reasons. (1) The venom was reported to evoke a quantity of m.e.p.p.s. limited to the calculated number of synaptic vesicles in a nerve terminal (Longenecker *et al.* 1970), and has since been used to estimate the total number of quanta remaining in a nerve terminal after a regimen of stimulation (Ceccarelli Hurlbut & Mauro, 1972). Such estimations depend on the assumption that replenishment of synaptic vesicles is blocked by venom treatment. Endocytosis is thought to be the initial step in leading to this replenishment (Heuser & Reese, 1973). (2) Endocytosis should occur in calcium-contain-

ing as well as calcium-free Ringer solution. The locations of plasmalemmal deformations would then be similar with and without calcium in the Ringer solution, which they are not. To explore this point further, however, it would be necessary to examine nerve terminals in both conditions at a variety of different times after treatment with venom, since the time course of the release in calcium-containing Ringer could differ from that in calcium-free Ringer. (3) A limited examination of thin-sectioned nerve terminals from muscles fixed during venom treatment in calcium-free Ringer suggests that such terminals contain too few coated invaginations to account for the number of plasmalemmal deformations occurring outside the active zone in comparable freeze-fractured terminals.

This is an important point to explore in greater detail because the occurrence of exocytosis outside of the active zone implies that fusion of synaptic vesicles with the plasmalemma does not depend on some special property of the plasmalemma at the active zone. The normal restriction of vesicle fusion to the active zone would therefore result from other factors, such as the close proximity of synaptic vesicles. It is also possible that the location of the depolarization-induced calcium influx is the main factor limiting exocytosis to the active zone. The slow rate of calcium diffusion in cytoplasm (Rose & Lowenstein, 1975) would contribute to this gradient if the calcium influx were limited to, or concentrated at, the active zone. Indeed, it has been suggested that the large particles which flank the ridges might be the sites of calcium channels (Heuser *et al.* 1974).

The putative ability of venom to form conductive channels in nerve terminal membranes also can account for the discontinuous 'volleys' of m.e.p.p.s. which arise from discrete portions of the nerve terminal (del Castillo & Pumplin, 1975). In fact, the conductance of a single channel induced by spider venom in a lipid bilayer is very high (4×10^{-10} mho) (Finkelstein *et al.* 1976). In the same experiments, over-all conductances of 8×10^{-10} mho per 800 μm^2 were measured. Since 800 μm^2 is a reasonable estimate of the surface area of a neuromuscular junction, a large over-all conductance could be produced by a small number of channels. Given an initial resting potential of 90 mV, such channels would allow approximately 10^8 cations/sec to flow in, sufficient to equilibrate the internal and external cation concentrations in, at the most, a few minutes. Thus, it is possible that the volleys seen in calcium-free Ringer (del Castillo & Pumplin, 1975) result from the formation of single venom channels. In calcium-free Ringer, the process is slower, presumably due to the necessary calcium displacement, and individual volleys are not distinguishable. If molecules of venom can enter lipid bilayers and create channels, they must span the membrane and would be expected to give rise to particles if the membrane were freeze-fractured. However, such particles in the nerve terminal

plasmalemma would be almost undetectable due to the very low concentration predicted by the previous analysis. In fact, we were unable to detect any particles attributable to insertion of venom into the plasmalemma.

Botulinum toxin abolished the formation of plasmalemmal deformations in response to electrical stimulation. This toxin also decreased the number of plasmalemmal deformations at the active zone induced by venom acting in calcium-containing Ringer although it had little effect on the venom action in calcium-free Ringer. Similarly, botulinum toxin prevented the increase in large plasmalemmal particles normally produced by electrical stimulation. These results indicate that botulinum toxin blocks exocytosis. This could be the result of inhibition of the normal depolarization-dependent calcium channel by the toxin. Although such an explanation would be attractive, bypassing these channels with calcium ionophores does not overcome the effects of botulinum toxin (Pumplin & Reese, 1976*a*, *b*; Kao, Drachman & Price, 1976; Cull-Candy *et al.* 1976). However, if botulinum toxin inhibits a step which occurs after the entry of calcium, it is difficult to see why this toxin does not markedly alter the effect of venom in calcium-free Ringer, since vesicle fusion under these circumstances still presumably depends on a net rise in the internal concentration of calcium.

We thank E. J. Schantz for gifts of botulinum toxin, B. Reese and F. D. Nolan for technical assistance, K. Pettigrew for help with statistics, J. Eskin for help in preparation of the manuscript, and R. Holz for helpful discussion.

REFERENCES

AKERT, K., PFENNINGER, K., SANDRI, C. & MOOR, H. (1972). Freeze-etching and cytochemistry of vesicles and membrane complexes in synapses of the central nervous system. In *Structure and Function of Synapses*, ed. PURPURA, D. P. & PAPPAS, G. D., pp. 67–86. New York: Raven.

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L. & STEINHARDT, R. A. (1967). The effect of sodium concentration on calcium movements in giant axon of *Loligo forbesi*. *J. Physiol.* **192**, 43*P*.

BOROFF, D. A., DEL CASTILLO, J., EVOY, W. H. & STEINHARDT, R. A. (1974). Observations of the action of type A botulinum toxin on frog neuromuscular junctions. *J. Physiol.* **240**, 227–253.

CECCARELLI, B., HURLBUT, W. P. & MAURO, A. (1972). Depletion of vesicles from frog neuromuscular junctions by prolonged tetanic stimulation. *J. cell Biol.* **54**, 30–38.

CECCARELLI, B., PELUCHETTI, D., GROHOVAZ, F. & IEZZI, N. (1976). Freeze-fracture studies on changes at neuromuscular junctions induced by black widow spider venom. In *Proc. 6th European Congress on Electron Microscopy*, vol. II, *Biol. Sci.*, pp. 376–378.

CLARK, A. W., MAURO, A., LONGENECKER, H. C., Jr. & HURLBUT, W. P. (1970). Effects of black widow spider venom on the fine structure of the frog neuromuscular junction. *Nature, Lond.* **225**, 703–705.

CULL-CANDY, S. G., LUNDH, H. & THESLEFF, S. (1976). Effects of botulinum toxin on neuromuscular transmission in the rat. *J. Physiol.* **260**, 177–203.

DEL CASTILLO, J. & ENGBAEK, L. (1954). The nature of the neuromuscular block produced by magnesium. *J. Physiol.* **124**, 370–387.

DEL CASTILLO, J. & KATZ, B. (1954). Changes in endplate activity produced by presynaptic depolarization. *J. Physiol.* **124**, 586–604.

DEL CASTILLO, J. & PUMPLIN, D. W. (1975). Discrete and discontinuous action of brown widow spider venom on the presynaptic nerve terminals of frog muscle. *J. Physiol.* **252**, 491–508.

DREYER, F., PEPER, K., AKERT, K., SANDRI, C. & MOOR, H. (1973). Ultrastructure of the 'active zone' in the frog neuromuscular junction. *Brain Res.* **62**, 373–380.

FINKELSTEIN, A., RUBIN, L. L. & TZENG, M.-C. (1976). Black widow spider venom: effect of purified toxin on lipid bilayer membranes. *Science, N.Y.* **193**, 1009–1011.

FRONTALI, N., CECCARELLI, B., GORIO, A., MAURO, A., SIEKEVITZ, P., TZENG, M.-C., & HURLBUT, W. P. (1976). Purification from black widow spider venom of a protein factor causing the depletion of synaptic vesicles at neuromuscular junctions. *J. cell Biol.* **68**, 462–479.

GRASSO, A. (1976). Preparation and properties of a neurotoxin purified from the venom of black widow spider (*Latrodectus mactans tredecimguttatus*). *Biochim. biophys. Acta* **439**, 406–412.

HARRIS, A. J. & MILEDI, R. (1971). The effect of type D botulinum toxin on frog neuromuscular junctions. *J. Physiol.* **217**, 497–515.

HEUSER, J. E. & REESE, T. S. (1973). Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. cell Biol.* **57**, 315–344.

HEUSER, J. E. & REESE, T. S. (1975). Redistribution of intramembraneous particles from synaptic vesicles: direct evidence for vesicle recycling. *Anat. Rec.* **181**, 374.

HEUSER, J. E., REESE, T. S. & LANDIS, D. M. D. (1974). Functional changes in frog neuromuscular junctions studied with freeze-fracture. *J. Neurocytol.* **3**, 109–131.

HOLLANDER, M. & WOLFE, D. A. (1973). *Non-parametric Statistical Methods*. New York: Wiley and Sons.

KAO, I., DRACHMAN, D. B. & PRICE, D. L. (1976). Botulinum toxin; mechanism of presynaptic blockade. *Science, N.Y.* **193**, 1256–1258.

KENDRICK, N. C., BLAUSTEIN, M. P., FRIED, R. C. & RATZLAFF, R. W. (1977). ATP-dependent calcium storage in presynaptic nerve terminals. *Nature, Lond.* **265**, 246–248.

LONGENECKER, H. E., Jr., HURLBUT, W. P., MAURO, A. & CLARK, A. W. (1970). Effects of black widow spider venom on the frog neuromuscular junction. *Nature, Lond.* **225**, 701–703.

LOWE, D. A., RICHARDSON, B. P., TAYLOR, P. & DONATSCH, P. (1976). Increasing intracellular sodium triggers calcium release from bound pools. *Nature, Lond.* **260**, 337–338.

MCCRONE, J. D. & NETZLOFF, M. L. (1965). An immunological and electro-phoretical comparison of the venoms of North American *Latrodectus* spiders. *Toxicon* **3**, 107–110.

ORNBERG, R. L. & SMYTH, T., Jr. (1976). Physiological mode of action of a presynaptic toxin isolated from black widow spider venom. Sixth Annual Meeting Soc. *Neurosci. Abs.* **113**, 11.

ORNBERG, R. L., SMYTH, T., JR. & BENTON, A. W. (1976). Isolation of a neurotoxin with a presynaptic action from the venom of the black widow spider (*Latrodectus mactans, Fabr.*). *Toxicon* **14**, 329–333.

PFENNINGER, K., AKERT, K., MOOR, H. & SANDRI, C. (1972). The fine structure of freeze-fractured presynaptic membranes. *J. Neurocytol.* **1**, 129–149.

PFENNINGER, K. H. & ROVAINEN, C. M. (1974). Stimulation and calcium-dependence of vesicle attachment sites in the presynaptic membrane; a freeze-cleave study of the lamprey spinal cord. *Brain Res.* **72**, 1–23.

PUMPLIN, D. W. & DEL CASTILLO, J. (1975). Release of packets of acetylcholine and synaptic vesicles elicited by brown widow spider venom in frog motor nerve endings poisoned by botulinum toxin. *Life Sci.* **17**, 137–142.

PUMPLIN, D. & W. MCCLURE, W. O. (1977). The release of acetylcholine elicited by extracts of black widow spider glands: studies using rat superior cervical ganglia and inhibitors of electrically stimulated release. *J. Pharmac. exp. Ther.* (In the Press.)

PUMPLIN, D. W. & REESE, T. S. (1976*a*). Membrane structure of synapses poisoned by spider venom and botulinum toxin. *Trans. Am. Soc. Neurochem.* **7**, 297.

PUMPLIN, D. W. & REESE, T. S. (1976*b*). Freeze-fracture study of transmitter release at neuromuscular junctions treated with brown spider venom, botulinum toxin and cation ionophores. Sixth Annual Meeting Soc. *Neurosci. Abs.* **1010**.

ROSE, B. & LOEWENSTEIN, W. (1975). Calcium ion distribution in cytoplasm visualized by aequorin: diffusion in cytosol restricted by energized sequestering. *Science, N.Y.* **190**, 1204–1206.

SIMPSON, L. L. (1971). *Botulinum Toxin in Neuropoisons – Their Pathophysiological Actions*, vol. 1, ed. SIMPSON, L. L. New York: Plenum.

SPITZER, N. (1972). Miniature end plate potentials at mammalian neuromuscular junctions poisoned by botulinum toxin. *Nature, New Biol.* **237**, 26–27.

THESLEFF, S. (1960). Supersensitivity of skeletal muscle produced by botulinum toxin. *J. Physiol.* **151**, 598–607.

EXPLANATION OF PLATES

PLATE 1

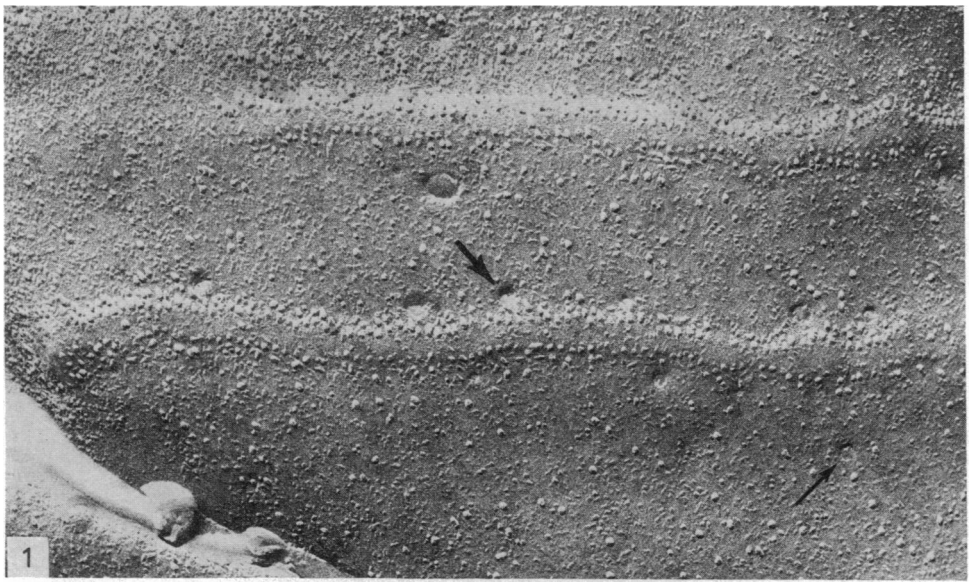
Fig. 1. Nerve terminal stimulated at 20 Hz in calcium. In this and succeeding figures the cytoplasmic half of the nerve terminal plasmalemma (A or P face) is viewed as if one were looking at the nerve terminal from a vantage point within the postsynaptic muscle fibre. Plasmalemmal deformations (large arrow) form in the active zone adjacent to the double row of large intermembrane particles lying on each side of parallel transverse ridges. Some plasmalemmal deformations (small arrow, lower right) are not in the active zone. × 100,000.

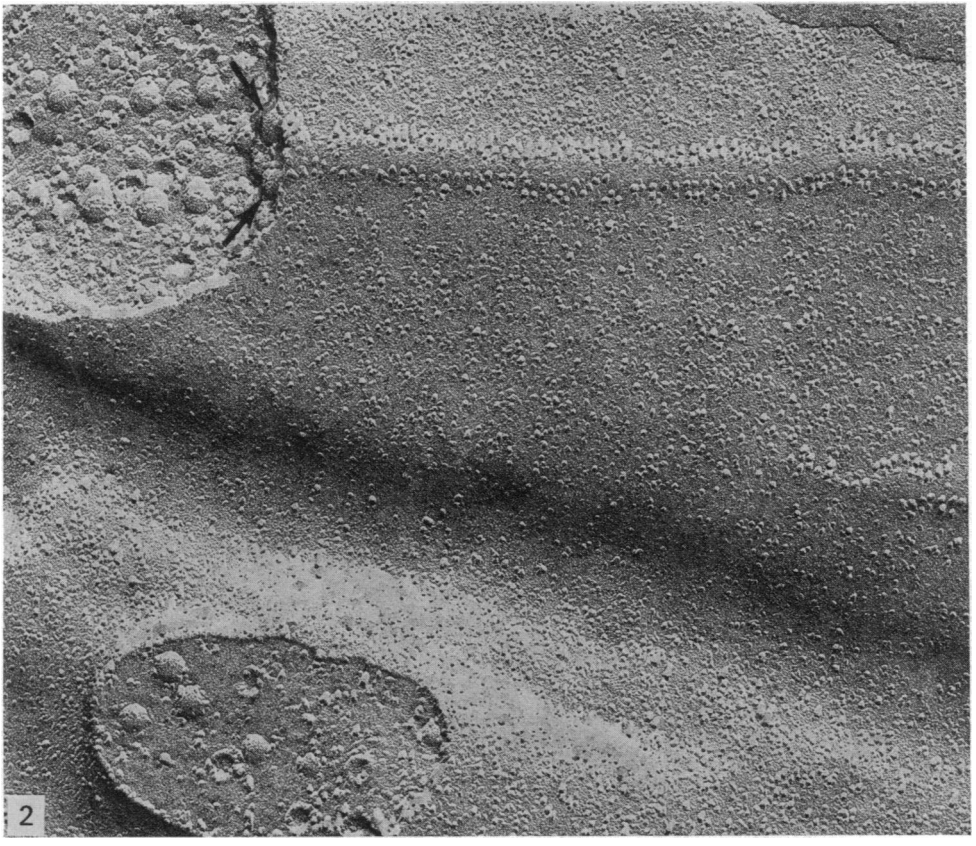
Fig. 2. Nerve terminal from botulinum toxin-treated muscle stimulated at 20 Hz in calcium. Plasmalemmal deformations are absent, although synaptic vesicles are lined up near the active zone in their usual position (arrows). × 100,000.

PLATE 2

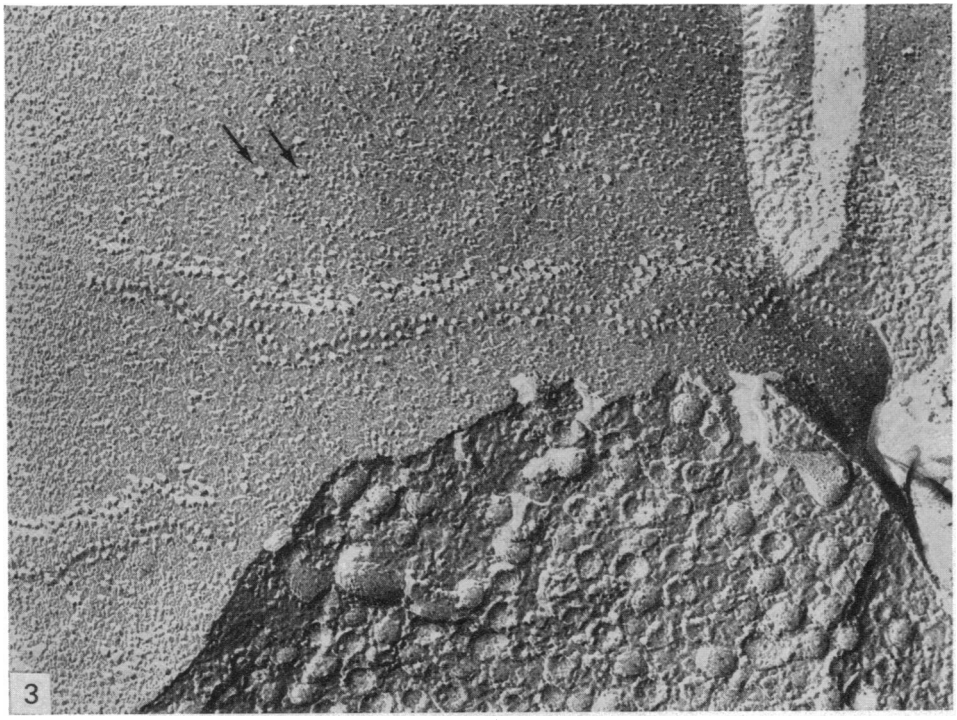
Fig. 3. Nerve terminal treated with brown widow spider venom at 1–2 °C. in calcium Ringer. Plasmalemmal deformations are absent and the cross-fractured portion of the terminal contains numerous synaptic vesicles (below). The concentration of large particles (arrows) in the plasmalemma is low compared to terminals treated in the same way at 20–22 °C (Pl. 2, fig. 4). × 100,000.

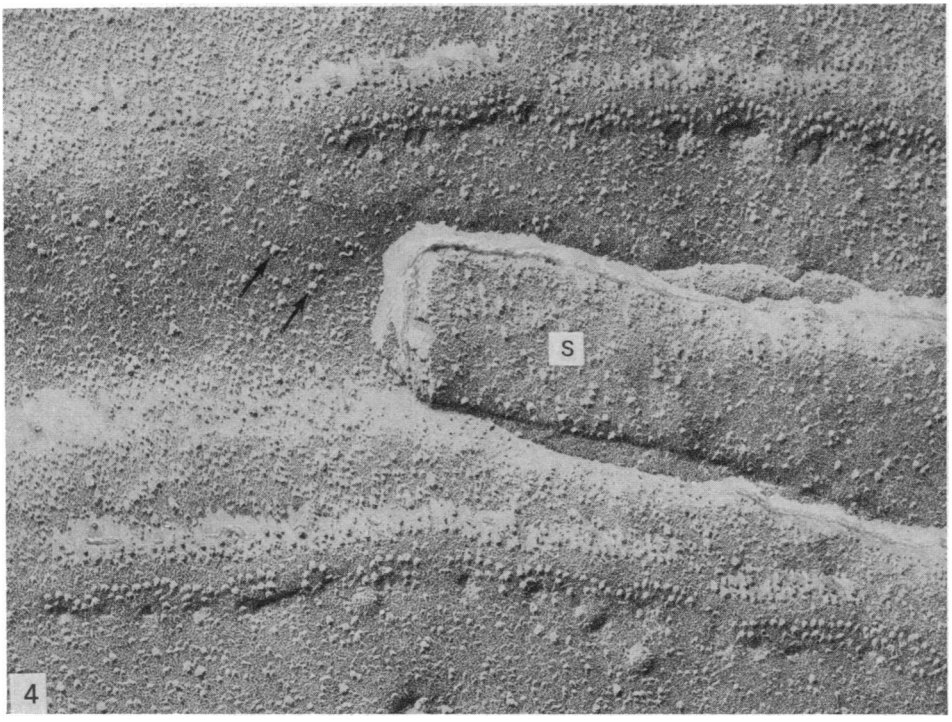
Fig. 4. Nerve terminal treated at 20–22 °C with brown widow spider venom in calcium Ringer. Plasmalemmal deformations occur almost exclusively in the active zones. The concentration of large plasmalemmal particles (arrows) is much larger than in fig. 3, where transmitter release was blocked. A process of a Schwann cell (S) lies between the active zones. × 100,000.





(*Facing p.* 456)


3


S
4


5


6

PLATE 3

Fig. 5. Nerve terminal treated at 20–22 °C with brown widow spider venom in calcium-free Ringer. Plasmalemmal deformations occur both in and away from (large arrow), the active zone. The concentration of large particles (small arrows) is also high. See Table 2 for their concentrations under a variety of experimental conditions. × 100,000.

Fig. 6. Nerve terminal from botulinum toxin-treated muscle treated at 20–22 °C with brown widow spider venom in calcium-free Ringer. Plasmalemmal deformations occur both in and away from (arrow), the active zone. See Table 3 for their frequencies under a variety of experimental conditions. × 110,000.